Original Article

The expression of *SALL4* is significantly associated with *EGFR*, but not *KRAS* or *EML4-ALK* mutations in lung cancer

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Background: Lung cancer is the leading cause of cancer-related deaths worldwide; unfortunately, its prognosis is still very poor. Therefore, developing the target molecular is very important for lung cancer diagnosis and treatment, especially in the early stage. With this in view, spalt-like transcription factor 4 (*SALL4*) is considered a potential biomarker for diagnosis and prognosis in cancers, including lung cancer.

Methods: In order to better investigate the association between the expression of *SALL4* and driver genes mutation, 450 histopathologically diagnosed patients with lung cancer and 11 non-cancer patients were enrolled to test the expression of *SALL4* and the status of driver genes mutation. This investigation included epidermal growth factor receptor (*EGFR*), kirsten rat sarcoma viral oncogene homolog (*KRAS*), and a fusion gene of the echinoderm microtubule-associated protein-like 4 (*EML4*) and the anaplastic lymphoma kinase (*ALK*).

Results: The results of the study showed that females harbored more *EGFR* mutation in adenocarcinoma (ADC). The mutation rate of *KRAS* and *EML4-ALK* was about 5%, and the double mutations of *EGFR/EML4-ALK* were higher than *EGFR/KRAS*. In the expression analysis, the expression of *SALL4* was much higher in cancer tissues than normally expected, especially in tissues that carried *EGFR* mutation ($P<0.05$), however, there were no significant differences between different mutation types. Likewise, there were no significant differences between expression of *SALL4* and *KRAS* and *EML4-ALK* mutations.

Conclusions: *SALL4* is up regulated in lung cancer specimens and harbors *EGFR* mutation; this finding indicates that *SALL4* expression may be relevant with *EGFR*, which could provide a new insight to lung cancer therapy. The mechanism needs further investigation and analysis.

Keywords: Epidermal growth factor receptor (*EGFR*); *EML4-ALK*; kirsten rat sarcoma viral oncogene homolog (*KRAS*); lung cancer; *SALL4* expression

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Introduction

Lung cancer is the most common type of cancer and is the leading cause of cancer-related deaths worldwide. An ever increasing development of detection and treatment exists; also an increasing number of lung cancer patients are diagnosed in early stage and have access to surgical resection. However, the prognosis is still very poor, for the 5-year survival rate is less than 20% worldwide, this 5-year rate is about 18% in China and even less than 10% in some other Asian countries (1). An overview of the various types of lung cancer can help clarify the various dimensions of this critically serious disease. There are two major types of lung cancer: (I) non-small cell lung cancer (NSCLC) and (II) small cell lung cancer (SCLC). The NSCLC accounts for about 85% of lung cancer diagnoses; in addition, this type

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of cancer contains three histologic types: adenocarcinoma (ADC), squamous cell carcinoma (SCC) and large cell carcinoma (LCC) (2).

Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) are typically used as the first-line treatment in the NSCLC patients who are found to harbor the *EGFR* mutation. *EGFR* mutations mainly occur in exon 18 to 21, which encode the tyrosine kinase domain, more mutations occur in AC, especially in non-smoking Asian females (3). *EML4-ALK* and *ROS1* are a two other genes of target therapy of lung cancer (4,5). Although target therapy could significantly increase survival and decrease undesirable side effects, drug resistant is often present. To improve the treatment of lung cancer, hopefully, more candidate genes which could be potential targets can be identified and used to both diagnose and offer more effective therapy.

Another factor in cancer, spalt-like transcription factor 4 (*SALL4*) (which is also known as *ZNF797*) belongs to the spalt family of zinc finger transcription factor and is required for early embryonic development (6,7). The most important function of *SALL4* is to maintain the properties of embryonic stem cells (ESCs) by interacting with other important molecules, such as Oct4 and Nanog (8-10). *SALL4* is required for DNA damage response in ESCs; it also maintains genomic stability during the expansion of ESCs (11). In human cancers, *SALL4* is also overexpressed, such as acute myelocytic and lymphocytic leukemia's, gastric cancer (12), glioma (13), and as well as in liver cancer. Up-regulation of *SALL4* associated with poor prognosis in many cancers (12,13), and *SALL4* expression were significantly correlated with gastric cancer cell metastasis to lymph nodes, especially in moderately differentiated tumor samples (14). Some investigators pointed out that serum *SALL4* could be used as a new biomarker for early cancer detection (15), tumor recurrence, and poor survival (16). *SALL4* is a potential, novel therapeutic target, but so far few investigations of *SALL4* focus on lung cancer, even in the early clinical stages (17). Further review has shown that *SALL4* expression is also significantly associated with drug-resistant. Up-regulated, the expression of *SALL4* could decrease sensitivity to anti-cancer drugs, such as cisplatin, carboplatin, and paclitaxel; this *SALL4* expression also may be involved in the recurrence of lung cancer after adjuvant chemotherapy (18). Further, no studies were found on the *SALL4* expression and driver genes mutation. This study was conducted to investigate the relationship between the expression of *SALL4* and driver genes mutation in lung cancer; therefore, 450 histopathologically diagnosed cases with lung cancer and 11 non-cancer patients were collected in this current study. The focus was to test, examine, and analyze the mutation status of *EGFR*, *KRAS*, *EML4-ALK*, and expression of *SALL4*.

Methods

Patients

In this study, all 450 lung cancer specimens were fresh tumor tissues obtained from the surgeries of lung cancer patients, and 11 normal lung tissues which were obtained from non-cancer patients; all people voluntarily joined this study with informed consent. For each case, the following medical records were gathered: clinical history, age, gender, cytologic diagnosis, and any subsequent histologic follow-up, if available. TNM (tumor, node, and metastases) staging was performed according the American Joint Committee for Cancer, 7th ed. (AJCC) staging system (19). The histologic subtypes of all patients were assessed and reassessed by at least two lung pathologists according to the 2011 International Association for the Study of Lung Cancer (IASLC)/American Thoracic Society (ATS)/European Respiratory Society (ERS) international multidisciplinary classification of lung ADC (20). The First Affiliated Hospital of Zhengzhou University ethics committee approved the protocol.

EGFR and KRAS mutation analysis

Extraction of DNA was performed first from fresh, surgically resected tumor samples. Next, DNA isolation was carried out using the AmoyDx Tissue DNA kit (Amoydx, Xiamen, China) according to the manufacturer's instructions. Then, a highly sensitive, real-time PCR-based AmoyDx *EGFR* 29 Mutation Detection Kit was used to accurately identify 29 *EGFR* mutations in exons 18–21. Similarly, AmoyDx *KRAS* 7 Mutation Detection Kit was used to detect 7 *KRAS* mutations in codon 12 and 13 [these two AmoyDx Detection Kits of tumor mutation are approved, respectively, by China Food and Drug Administration (CFDA) for clinical use in China and Conformite Europeenne (CE) marked for in vitro diagnostic (IVD) products used in Europe].

EML4-ALK rearrangement analysis

Extraction of RNA was performed first from fresh, surgically resected tumor samples. Next, RNA isolation was

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done with the AmoyDx Tissue RNA kit (Amoydx, Xiamen, China) according to the manufacturer's instructions. Then, *EML4-ALK* rearrangement was detected with AmoyDx *EML4-ALK* Gene Detection Kit (Amoydx, Xiamen, China) according to the manufacturer's instructions. Briefly, first strand cDNA was synthesized from RNA using moloney murine leukemia virus (M-MLV) reverse transcriptase (Amoydx, Xiamen, China). The total amount of RNA should be within 0.1–5 μg, and A260/A280 value should be between 1.9 and 2.1. Target gene sequence was specifically amplified by proprietary primers. The amount of target cDNA was measured after each cycle in the data capture phase using a fluorescent probe. The fusion status of each sample and reference gene expression status were indicated by the FAM fluorescent signal.

SALL4 expression detection

First strand cDNA was synthesized from 1 μg total RNA using M-MLV reverse transcriptase (Amoydx, Xiamen, China) and downstream primers of the quantificational real-time polymerase chain reaction (qRT-PCR) primers as the anchor primer. The qRT-PCR primers were designed using Beacon Designer 7 (Premier Biosoft International, Palo Alto, Calif., USA). The primers of *SALL4* are designed across introns. The reaction mixtures were incubated at 95 ℃ for 5 min followed by 42 ℃ for 1 h and 4 ℃ continuously.

The qRT-PCR reactions were performed using Mx3000P (Agilent, USA). The components of qPCR reaction buffer were 0.08 μL of SYTO9 working liquid, 25 mM of dNTPs, 10 mM of each primer, 2.5 mM of $MgCl_2$, 5 U/μL of Taq DNA polymerase (Amoydx, Xiamen, China) in a total volume of 20 μL. The reaction mixture consisted of 2.5 μL of cDNA template. 18SrRNA was as internal reference.

The qRT-PCR protocol included an initial step of 95 ℃ for 5 min, followed by 45 cycles of 95 ℃ for 25 s and then annealed at 60 ℃ for 40 s, followed by one cycle of 95 ℃ for 1 min, 55 ℃ for 30 s, and 95 ℃ for 30 s. qPCR amplicons were subjected to melting curve analysis. The specificity of the qRT-PCR reactions was monitored with melting curve, analyzing by MxPro3000P and gel electrophoresis.

Statistical analysis process

The association of genes with clinical and pathologic characteristics was tested by Fisher's exact test, and the independent t test was applied to continuous data. All statistical analysis was performed by using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). All P values were based on a two-sided hypothesis. The statistical significance was set at $P<0.05$ for all analyses.

Results

Clinical characteristics and driver genes mutation status of samples

In order to investigate the association between driver genes mutation and expression of *SALL4*, we recruited 450 cases histopathologically diagnosed with lung cancer to carry out this study. We have listed the clinical characteristics of the patients (*Table 1*). More males were in the study, and the mean age is 61.5±9.2; more patients were over 61 years old. About 62.7% of the samples were in grade I, and about 20% of the patients were diagnosed as "positive" in their lymph nodes. The largest type we collected was ADC, followed by SCC. The two types accounted for about 82.4% of lung cancer samples. The status of *EGFR*, *KRAS*, and *EML4-ALK* were also listed (*Table 1*). The mutation rate of *EGFR* is about 47.7% in all lung cancer patients. L858R and 19-del are the main mutation type of *EGFR*, and account for about 23.6% and 18.0%, respectively. The mutation rate of *KRAS* and *EML4-ALK*, collectively, amounted to about 5%.

Clinical characteristics and driver genes mutation status in EGFR mutation samples

Because of increased mutations in *EGFR*, clinical information of *EGFR* mutation group was collected (*Table 2*). More females demonstrated *EGFR* mutation, accounting for 56.8%. The mean age of patients who harbored *EGFR* mutations was 60.9±9.3. About 67.6% patients were grade I, and about 21.6% patients were diagnosed as "positive" in their lymph nodes. About 87.3% *EGFR* mutations were in ADC and 2.3% in SCC. The results also showed that (I) two patients harbored both *EGFR* mutation and *KRAS* mutation; (II) eight patients carried both *EGFR* mutation and *EML4-ALK* rearrangements.

Association between SALL4 expression and driver gene mutation

In order to analyze the association between driver gene mutation and the expression of *SALL4*, specimens were collected from 450 lung cancer patients and 11 controlled group patients to determine the mutation status of *EGFR*,

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Table 1 Clinical characteristics and driver genes mutation of patients

Characteristic	N=450	%
Gender		
Male	274	60.9
Female	176	39.1
Age (years) (mean ± std)	61.5±9.2	
≤61	207	46
>61	243	54
Pathological stage		
I	282	62.7
II	46	10.2
III	93	20.7
IV	29	6.4
Lymph nodes		
Positive	90	20
Negative	294	65.3
Unknown	66	14.7
Histologic diagnosis		
Atypical adenomatous hyperplasia	4	0.9
Adenocarcinoma	285	63.3
Large-cell carcinoma	12	2.7
Adenosquamous carcinoma	4	0.9
Squamous-cell carcinoma	86	19.1
Mesothelioma	5	1.1
Small cell lung cancer	2	0.4
Others	52	11.6
Type of *EGFR* mutation		
Wild type	237	52.6
19-del	81	18
L858R	106	23.6
Others	26	5.8
KRAS mutation		
Mutation	21	4.7
Wild type	429	95.3
ALK rearrangement		
Mutation	23	5.1
Wild type	427	94.9

std, standard deviation; *EGFR*, epidermal growth factor receptor; *KRAS*, kirsten rat sarcoma viral oncogene homolog; *ALK*, anaplastic lymphoma kinase.

Table 2 Clinical characteristics and driver genes mutation of patients carried *EGFR* mutation

Characteristic	No. of patients (%)	
	EGFR mutation	%
Sex		
Male	92	43.2
Female	121	56.8
Age (years) (mean ± std)	60.9±9.3	
≤61	102	47.9
>61	111	52.1
Pathological stage		
I	144	67.6
II	12	5.6
III	42	19.7
IV	15	7.1
Lymph nodes		
Positive	46	21.6
Negative	146	68.5
Unknown	21	9.9
Histologic diagnosis		
Atypical adenomatous hyperplasia	2	0.9
Adenocarcinoma	186	87.3
Large-cell carcinoma	2	0.9
Adenosquamous carcinoma	4	1.9
Saquamous-cell carcinoma	5	2.3
Mesothelioma	0	0
Small cell lung cancer	1	0.5
Others	13	6.1
KRAS mutation		
Mutation	2	0.9
Wild type	211	99.1
ALK rearrangement		
Mutation	8	3.8
Wild type	205	96.2

std, standard deviation; *EGFR*, epidermal growth factor receptor; *KRAS*, kirsten rat sarcoma viral oncogene homolog; *ALK*, anaplastic lymphoma kinase.

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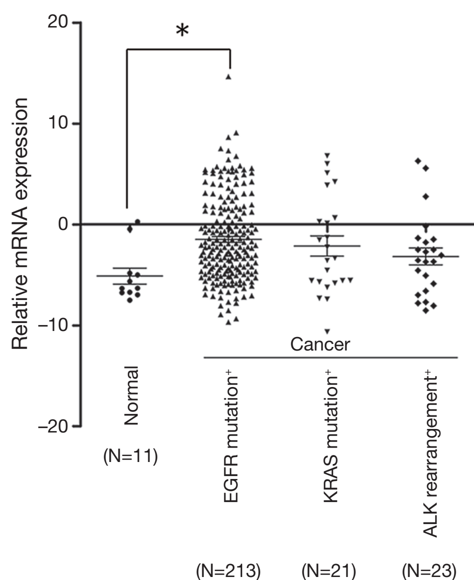

Figure 1 The expression of *SALL4* between normal tissues and tumor tissues. The tumor tissues harbored *EGFR*, *KRAS*, and *EML4-ALK* mutation. There was significantly difference between *EGFR* mutation tumors and normal tissues. *, means P<0.05. *SALL4*, spalt-like transcription factor 4; *EGFR*, epidermal growth factor receptor; *KRAS*, kirsten rat sarcoma viral oncogene homolog; *EML4-ALK*, a fusion gene of the echinoderm microtubule-associated protein-like 4 and the anaplastic lymphoma kinase.

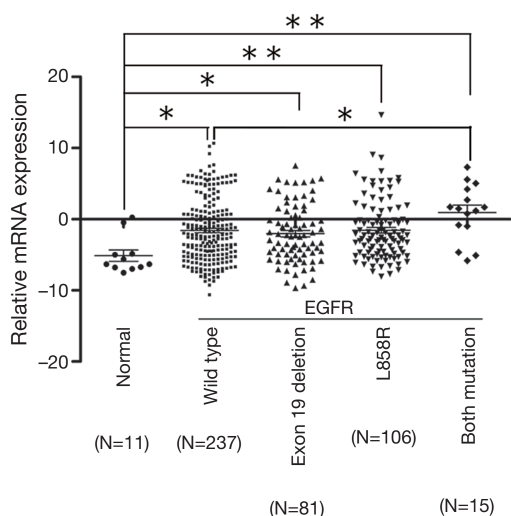

Figure 2 The expression of *SALL4* between normal tissues and *EGFR* mutation tumor tissues. The mutation types include deletion of exon 19 (19-del), L858R, and these two mutations. There was significant difference between normal tissues and *EGFR* mutation tissues. *, means P<0.05; **, means P<0.01. *SALL4*, spalt-like transcription factor 4; *EGFR*, epidermal growth factor receptor.

KRAS, *EML4-ALK* and the expression of *SALL4* (*Figure 1*). Compared to the control group, the expression of *SALL4* in patients who harbored driver genes mutation was higher, but only *EGFR* mutation presented significant difference (P<0.05).

Association between SALL4 expression and EGFR mutation

In order to further investigate the association between the expression of *SALL4* and *EGFR* mutation, two major mutation types were selected, L858R and 19-del, and a rare type which carried both L858R and 19-del mutation (*Figure 2*). Significant differences occurred between controls and cases in the expression of *SALL4*. The higher expression was present in all types of *EGFR*, including wild type, L858R, 19-del, and both mutations (P<0.05). An interesting result was that the expression of *SALL4* in the patients who harbored two mutations was much higher than other mutation types of *EGFR* (P<0.05); however, no significant differences between other mutation types surfaced in the results.

Discussion

Lung cancer is the most common type of cancer, and is the leading cause of cancer-related deaths worldwide. Finding a useful, therapeutic target is very meaningful for lung cancer treatment. *SALL4* is considered a potential therapeutic target in cancer, but our review found few investigations on lung cancer. In the current study, we analyzed the association between *SALL4* expression and mutant status of driver genes, including *EGFR*, *KRAS*, and *EML4-ALK*. The results showed that *SALL4* is overexpressed in lung cancer, and the expression was significantly associated with *EGFR* mutation.

The mutations of *EGFR* mainly located in exon 18–21, which encode the tyrosine kinase domain in lung cancer. The significant association between *EGFR* mutations and the treatment of NSCLC was first described in 2004 (21,22). The two most common mutations are L858R in exon 21 and deletion in exon 19. L858R is higher than 19-del, 21% and 19%, respectively (23). Our study showed similar results: L858R mutation is about 23.6%, 19-del is about 18%, and the mutation which contained both L858R and 19-del is about 3.3%. These two mutations are sensitive to TKIs (24). The mutant rate of *EGFR* is about 47.7% in lung cancer, and about 74.7% in ADC. In the mutation analysis, 56% of the female carried *EGFR*

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mutation, and about 87.3% of the mutation was in ADC. This is accordance with a previous report that the mutation of *EGFR* is present in never-smoker Asian female with ADC (3). *KRAS* mutation differs between a westerner and an Asian, for the occurrences of *KRAS* are about 21% and 5–11%, respectively (25). Our data showed that about 5% of the patients harbored *KRAS* mutation, which was similar to this report. In addition, *EML4-ALK* is another important target in lung cancer therapy (5). The rearrangement of *EML4-ALK* is about 4–7% in NSCLC (25). Our study also detected about 5% lung cancer patients carried *EML4-ALK* fusion gene. Our analysis found 0.9% patients carried both *EGFR* and *KRAS* mutations, and 3.8% patients carried *EGFR/EML4-ALK* mutations.

SALL4 is over-expressed in many cancers, such as lymphocytic leukemia, gastric cancer and glioma; however, few studies investigated this relationship to ones who had lung cancer. Some former studies pointed out that only a small proportion of lung cancer positively expressed *SALL4* (26,27), and there were differences between ADC and SCC (26). Another study showed that about 93% of samples presented, there was a more than two-fold change of *SALL4* expression, and *SALL4* was highly expressed even in early clinical stages (17). Our study also showed that the expression of *SALL4* is higher in tumor tissues than normal tissues, and even about 80% of samples expressed greater than a two-fold change; there was, however, no significant difference between ADC and SCC (data not shown).

The results of the analysis of the association of *SALL4* expression with *EGFR*, *KRAS* and *EML4-ALK* mutations were striking; these showed that compared to normal tissues, overexpression of *SALL4* was detected in tumor tissues which harbored *EGFR*, *KRAS* and *EML4-ALK* mutations. The less positive tumor tissues in *KRAS* and *EML4-ALK* demonstrated insignificant differences. However, the *EGFR* mutation group showed significant results. Further analysis made with a different *EGFR* mutation showed us that *SALL4* is up regulated in cancer tissues. However, there were no significant differences between wild type, L858R, and 19-del group. The most significant result was found in tissues which harbored both L858R and 19-del mutations. We believe our results indicate a more ample supply of tissues is needed to further confirm the data.

Finally, the *SALL4* is up-regulated in lung cancer specimens with *EGFR* mutation indicates that the expression of *SALL4* may be relevant with *EGFR* mutation. This conclusion could provide a new insight to lung cancer therapy. Therefore, further investigation of this mechanism is deemed to be much worthwhile.

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None.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The First Affiliated Hospital of Zhengzhou University ethics committee (approval number: research-2015-20) approved the protocol. Written informed consent was obtained from all patients.

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